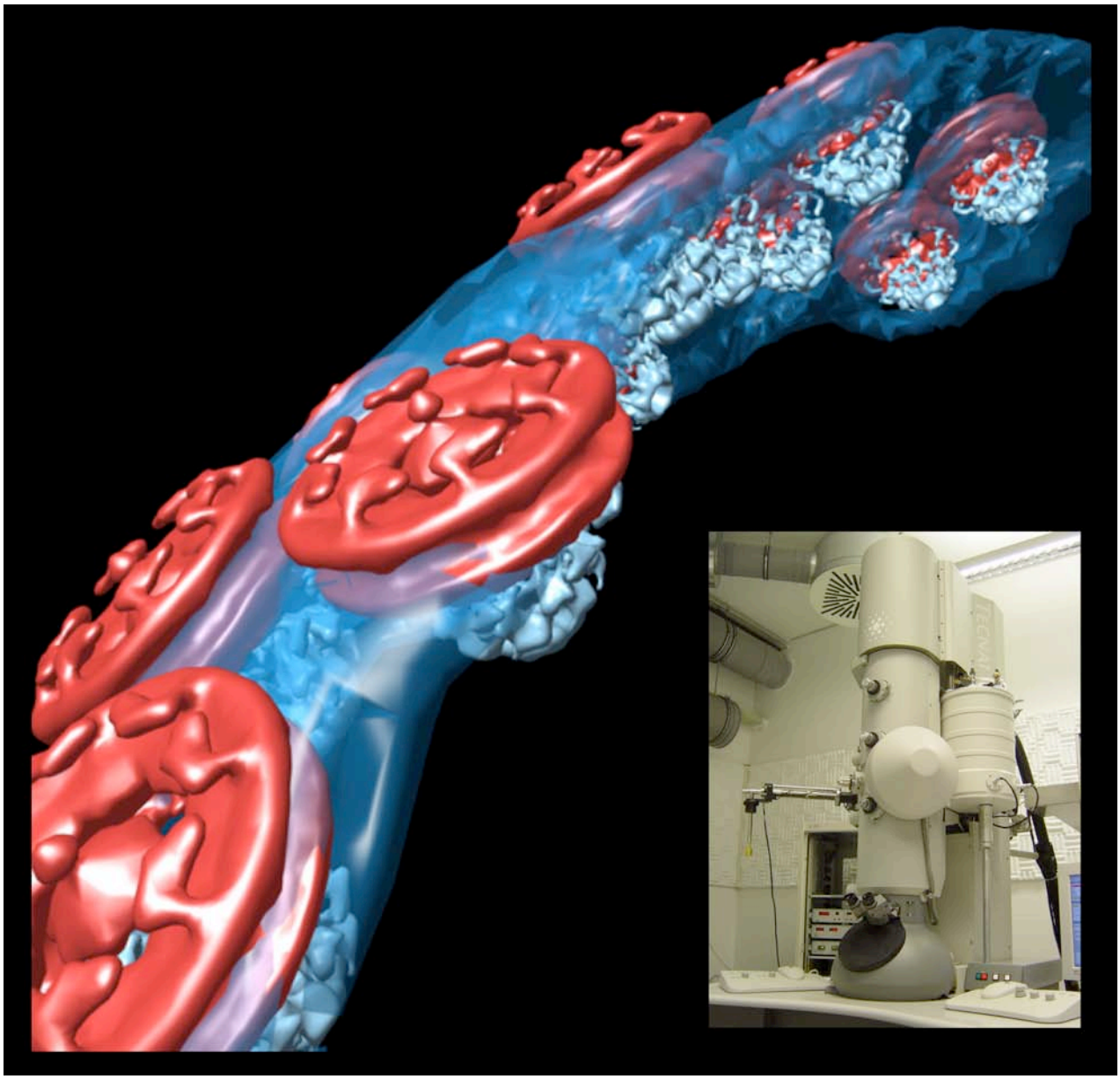
Forum for European Structural Proteomics

Electron Microscopy



FESP Report on EM

Introduction

With the development of methods for three-dimensional reconstruction and of specimen preparation techniques allowing the study of biological materials in a close-to-life state, electron microscopy has become a major player in structural biology. There is a broad consensus amongst its practitioners that electron microscopy has not yet realized its full potential, and that further advances in methodology and technology would open up new opportunities for studying complex biomolecular systems, both *in vitro* and *in situ*. This would enable scientists to explore largely uncharted territories, such as the supramolecular organization inside organelles or cells [1]. At the same time, it would strengthen European competitiveness in an important industrial sector, namely the manufacturing of instruments for nanoscale imaging.

The role of electron microscopy in structural biology

Over the past decade, cryo-electron microscopy has increasingly replaced the traditional methods of sample preparation (plastic embedding, negative staining) which were notoriously prone to artifacts. Cryo-electron microscopy is a generic term that refers to various electron-microscopic imaging modalities when applied to samples embedded in amorphous ice. Samples are vitrified by plunge freezing or high-pressure freezing and examined without chemical fixation or staining. Three major branches of cryo-electron microscopy are relevant in the context of molecular structural biology: Electron crystallography, single-particle analysis and electron tomography.

Electron crystallography relies on the availability of two-dimensional crystals, either natural or synthetic. It is particularly suited to studying membrane proteins, but its use is not restricted to this class of protein. Very high resolution (< 3 Å) can be attained by careful optimization of the imaging conditions and by applying image processing strategies which allow compensation for imperfections in the crystal lattices. Data acquisition can be very time consuming because of difficulties in collecting data sets of consistent quality; image quality

is often degraded, particularly at high tilt angles, for reasons that are not fully understood at present. Currently, very large data sets have to be recorded of which only a tiny subset is eventually used. This is a wasteful procedure, and could be avoided if automated screening methods were developed and implemented that would allow selection of (2-D) crystals diffracting to high resolution. Other problems that are in need of in-depth investigation are crystal flatness and charging effects.

Single particle analysis – a misleading name – relies on the existence of multiple copies of the object. Molecules suspended in thin layers of ice occur in random orientations. After grouping them into classes that correspond to common orientations, class averages are generated. Three-dimensional reconstructions are obtained by assigning relative orientations to the class averages and placing them in a virtual tilt experiment. Single-particle analysis is particularly suited to studying macromolecular complexes – the larger the better. Some degree of heterogeneity in the sample (for example variations in subunit composition, stoichiometry or conformational states) is tolerable, and can be taken into account by image classification. There is no fundamental reason why atomic resolution could not be attained, but hitherto this has remained an elusive goal. Medium resolution (1-2 nm) maps are obtained routinely, but subnanometer resolution maps require major efforts both in image acquisition and analysis. Medium-resolution maps are usually adequate for fitting of high-resolution structures of components (subunits, domains) determined by x-ray crystallography or NMR spectroscopy into the cryo-electron microscopy maps of the complex. At subnanometer resolution, elements of secondary structure can be discerned, enabling the docking of partial structures to be carried out with high accuracy. But in any case the docking should not be done in an *ad hoc* manner but should follow optimized procedures providing figures of merit for the quality of the fit. Efforts are in progress, particularly in the US, to increase the speed of single-particle techniques, by automated data acquisition and image analysis. There is also a need to develop more powerful image classification tools for dealing with structurally heterogeneous populations of molecules [2].

Electron tomography is unique in its capability to provide three-dimensional reconstructions of non-repetitive structures. Therefore, it enables insights into the molecular organization of higher-order structures displaying some degree of stochasticity. Objects are reconstructed from a series of transmission electron micrographs taken from different viewing angles.

During data collection, the requirement for optimal sampling must be reconciled with the need to avoid radiation damage (through keeping the cumulative electron dose low). Tomograms taken under these conditions are rich in information, but their poor signal-to-noise ratio makes interpretation difficult. Tomograms of intact cells or organelles are images of their entire proteomes, and sophisticated pattern-recognition methods must be applied in order to make use of the information. At the current resolution of 4-5 nm, typically obtained with intact cells, only large complexes can be visualized and mapped with an acceptable fidelity. With advances in instrumentation, resolutions of 2-3 nm are a realistic goal, and will enable cells to be mapped in a comprehensive manner ('visual proteomics'). Both instrumental improvements and better image processing tools will be needed to fully realize the enormous potential of electron tomography for bridging the gap that currently divides molecular and cellular structural studies [3].

What is needed to realize the full potential of cryo-electron microscopy?

As indicated in the previous paragraph, several advances in instrumentation are needed to achieve our goals. Here we should capitalize on the fact that two out of four manufacturers of transmission electron microscopes (FEI Company and Zeiss) are based in Europe; the two others (Hitachi and Jeol) are located in Japan. Europe has a very strong tradition in the development and manufacturing of electron microscopes; after all, the first electron microscope was built by Ernst Ruska (Berlin) in the 1930s. Nevertheless, industry is often hesitant to embark on new developments, and it is usually the scientific community that drives these developments. This can and should happen in a partnership between academia and industry, and the EC could play a critical role in fostering and supporting this partnership by funding joint projects which would include not only the major industrial players but also SMEs.

In several rounds of discussions organized by FESP a consensus emerged regarding the needs of the scientific community. First and foremost, there is an urgent need for better detectors. Basically all high-end cryomicroscopy is done with intermediate voltage (200-400 keV) electron microscopes; at those voltages the performance of today's CCD cameras (their detective quantum efficiency) is very poor. A factor 3-5 could be gained by the use of more

sensitive (and radiation hard) detectors – which currently do not exist. This alone would improve resolution in electron tomography by a factor of two. Therefore, the development of better detectors is of the highest priority, the more since there is ample knowhow in Europe in detector technology.

Electron tomography hinges upon the performance of goniometers for tilting. Ideally, they should be eucentric, and should allow tilting around multiple axes in order to minimize the effects of missing data. Significant improvements over currently available dual-axis tilt goniometers are highly desirable in order to permit dual-axis tilt experiments to be performed routinely. Devices which would allow introduction of multiple samples into the electron microscope for sequential examination ('autoloaders') could speed up data collection substantially.

For the examination of thicker (> 500 nm) samples, use of energy filters for removal of the adverse contributions of inelastically scattered electrons offers significant advantages. Ideally, they should be of the 'in-column' and not of the 'post-column' type. This greatly simplifies their integration into the microscope control software and offers more flexibility. Currently, two somewhat controversial issues are the advantages of liquid-helium over liquid nitrogen specimen temperatures, and the use of zone plates in conjunction with aberration correction for contrast enhancement. But both issues are of sufficient interest to deserve in-depth investigations.

An emerging field is correlative microscopy, enabling the examination of one and the same sample by both light and electron microscopy. Light microscopy provides a survey over large cellular landscapes, and the use of fluorescence microscopy allows positive identification of features of interest. Cryo-electron tomography in a correlative mode then permits zooming in on such features, and performance of in-depth analysis at much higher resolution. The development of instrumentation for correlative microscopy is of the utmost importance for addressing a wide range of biological problems, and has undoubtedly a large commercial potential.

Of great importance is the development of software for image analysis and for visualization. Europe has a number of very strong groups in academia developing relevant software, but

their efforts are not very well coordinated. As a consequence, many of the (fragmental) software packages are not particularly user-friendly, are poorly documented, and little or no support is available when problems are encountered. This makes it very difficult for non-experts to use them, and increases the risk of making mistakes. In other words, creation of a user-friendly image-processing platform would be an invaluable service to the scientific community.

Moreover, there are some particular needs for the development of more powerful software tools: In single-particle analysis there is a need for automation of the processing of very large data sets, and for more powerful image classification tools to deal with heterogeneous populations of molecules In electron tomography, to take full advantage of the imposing amount of information contained in the tomograms, there is a need for advanced pattern recognition methods for their molecular interpretation. Also the segmentation of tomograms, which is currently done manually by most investigators, should be automated in such a manner as to make the procedure more objective and reproducible.

Some organizational matters and recommendations

Currently, the situation with regard to high-end instrumentation in Europe as a whole is not too bad. In fact, it is comparable, in terms of the number of instruments, with that in the USA. However, there are regional differences, and there is basically no infrastructure of this kind in either Southern or Eastern Europe, even though some of the countries in both regions possess the expertise to run such instruments (*e.g.* Spain, Czech Republic).

The costs of instruments for cutting edge research in biomolecular electron microscopy are high, and are increasing steadily. Today, a 300-400 keV field emission instrument costs approx. € 3 Mio, and equipping it with large area detectors (e.g. 8kx8k), energy filters, autoloaders etc., adds substantially to the price. Moreover, one instrument does not make a center; therefore, an investment of approx. € 10 Mio is needed to establish one. And it is quite foreseeable that in the next five years, with the developments under way, the price of instrumentation will go up substantially. Maintenance costs are a major burden even for well-funded institutions; for a center they can be expected to amount to approx. € 500.000 per

annum. Therefore, to provide access to high-end infrastructure it will be necessary to create regional centers, and the EU should not restrict its support to providing access. After all, the infrastructure must be there, in the first instance, before access can be provided. Models should be developed in the context of ESFRI projects such as INSTRUCT or the Era-Net Initiative, and it is recommended that 5-10 such regional centers should be established across Europe. Unfortunately, Europe does not, as yet, have a culture of 'big grants' for establishing and running the equivalent of National Centers in the USA.

Another problem is the level of training. Electron optics used to be a major activity in European applied physics institutes, but this tradition has been discontinued in many cases. As a result, it is currently almost impossible to find scientists or engineers with a thorough understanding of electron optics. The problem is the same in Japan, where it has recently been brought to the attention of the national science council. This affects not only the development potential of the manufacturers of electron optical instrumentation but also the quality of servicing of the instruments. Poor maintenance of instruments is a reason why many laboratories which have purchased high-end instruments underachieve. Therefore, training programs should be established in Europe, ideally in a close collaboration between academic institutions and industry.

Finally, many of the methods and instrumentation developments required to take full advantage of the potential of cryo-electron microscopy are of a long-term nature, and should not be expected to deliver biological output within two or three years. Nevertheless, researchers must be empowered to pursue long-term objectives based on a clear vision, and to take on the necessary challenges without feeling threatened by the risk of losing their funding.

References

[1] *Robinson, C.V., A. Sali and W. Baumeister:* The molecular sociology of the cell. Nature Insight Review. *Nature* **450**, 973-982 (2008).

[2] *Sali, A., R. Glaeser, T. Earnest and W. Baumeister:* From words to literature in structural proteomics. *Nature* **422**, 216-225 (2003).

[3] *Nickell, S., C. Kofler, A. Leis and W. Baumeister:* A visual approach to proteomics. *Nat. Rev. Mol. Cell Biol.* **7**, 225-230 (2006).